A Novel Genomic Signature with Translational Significance for Human Idiopathic Pulmonary Fibrosis

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Abstract

The bleomycin-induced rodent lung fibrosis model is commonly used to study mechanisms of lung fibrosis and to test potential therapeutic interventions, despite the well recognized dissimilarities to human idiopathic pulmonary fibrosis (IPF). Therefore, in this study, we sought to identify genomic commonalities between the gene expression profiles from 100 IPF lungs and 108 control lungs that were obtained from the Lung Tissue Research Consortium, and rat lungs harvested at Days 3, 7, 14, 21, 28, 42, and 56 after bleomycin instillation. Surprisingly, the highest gene expression similarity between bleomycin-treated rat and IPF lungs was observed at Day 7. At this point of maximal rat–human commonality, we identified a novel set of 12 disease-relevant translational gene markers (*C6*, *CTHRC1*, *CTSE*, *FHL2*, *GAL*, *GREM1*, *LCN2*, *MMP7*, *NELL1*, *PCSK1*, *PLA2G2A*, and *SLC2A5*) that was able to separate almost all patients with IPF from control subjects in our cohort and in two additional IPF/control cohorts (GSE10667 and GSE24206). Furthermore, in combination with diffusing capacity of carbon monoxide measurements, four members of the translational gene marker set contributed to stratify patients with IPF according to disease severity. Significantly, pirfenidone attenuated the expression

change of one (*CTHRC1*) translational gene marker in the bleomycin-induced lung fibrosis model, in transforming growth factor- β 1-treated primary human lung fibroblasts and transforming growth factor- β 1-treated human epithelial A549 cells. Our results suggest that a strategy focused on rodent model–human disease commonalities may identify genes that could be used to predict the pharmacological impact of therapeutic interventions, and thus facilitate the development of novel treatments for this devastating lung disease.

Keywords: biomarkers; genomics; lung fibrosis; animal model

Clinical Relevance

Our genomics studies will help to support the optimal use of the bleomycin model on the basis of treatment timing and molecular analysis of translational pathways. Furthermore, this gene set could also find application as a potential diagnostic and prognostic tool for idiopathic pulmonary fibrosis in clinical practice.

Idiopathic pulmonary fibrosis (IPF) is the most severe and most common form of all idiopathic interstitial pneumonias. It is

a chronic and progressive lung disease with poor prognosis that affects roughly 5 million people worldwide (1). The disease affects

men more frequently than women, with an average onset at over 66 years of age and median 3- to 5-year mortality rates of

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approximately 50% without lung transplantation (2). Clinical disease manifestations include dyspnea and gradual worsening of lung function, although considerable heterogeneity in progression rate is observed. The diagnosis of IPF is based on lung function measurements, high-resolution computer tomography, surgical lung biopsy, which shows a typical histological appearance of usual interstitial pneumonia (UIP), and exclusion of other causes of interstitial lung disease (3). UIP features alternations of normal lung structures and regions of dense fibrosis, whereas signs of inflammation are usually mild. The presence of infiltrating lymphocytes and macrophages has been reported (4). The disease etiology remains unknown, but it is thought to involve chronic epithelial lung injury, myofibroblast formation, and excessive deposition of extracellular matrix in the lung parenchyma (5–10).

Recent genomic studies using surgical biopsies from patients with IPF support these findings, and have, in addition, demonstrated pathological gene expression changes in processes such as coagulation, angiogenesis, inflammation, apoptosis, and regeneration (11–15). These studies revealed a number of possible pathological pathways, and helped to identify candidate biomarkers that could be used for early diagnosis, prognosis, and disease progression monitoring (16–20). However, despite the significant increase in our understanding of the basic mechanisms of lung fibrosis, most clinical studies failed to show significant benefit, and only pirfenidone (Esbriet) was approved in selected countries for treatment of IPF, but not in the United States (21). Because most of the drugs that were ineffective in humans were efficacious in rodent models of bleomycin-induced fibrosis, it was suggested that the lack of suitable animal models with sufficient similarity to IPF, and the lack of translational markers, prevented successful drug discovery. Although a variety of animal lung fibrosis models have been developed, the bleomycin-induced lung fibrosis model remains the most frequently applied (22). In most cases, bleomycin is instilled intratracheally to induce a multiphasic process that starts with a pronounced inflammatory response followed by a fibrotic process. The resulting excessive

deposition of extracellular matrix and lung remodeling activity lead to histological changes, such as obliteration of the alveolar space, histiocytosis, and septal collagen accumulation, which are, to some extent, similar to changes seen in biopsies from patients with IPF (23, 24). However, the molecular similarities with the human disease and their relevance to disease progression have not been sufficiently assessed.

We analyzed genome-wide expression changes in a bleomycin-induced rat lung fibrosis time course model, and compared them to gene expression signatures obtained from a large collection of IPF lungs to identify the time point of highest similarity between the rodent model and the human disease. This approach led to the identification of a novel disease-relevant translational gene marker set that separated IPF-derived samples from controls and identified genes that allowed stratification of patients with IPF according to disease severity. Finally, pirfenidone attenuated the expression changes of two members (*Fhl2* and *Cthrc1*) of the translational gene marker set in the bleomycin-induced lung fibrosis model, and the transforming growth factor (TGF)- β 1-induced expression change of *CTHRC1* was attenuated in primary human lung fibroblasts and human epithelial A549 cells. This suggests that this novel disease-relevant gene panel could be used to investigate new treatment modalities and to explore combination therapies with improved efficacy.

Materials and Methods

Patients

Genomic and clinical data from human lung tissues of patients with IPF (71 males, 29 females) or normal histology control subjects (49 males, 59 females) were obtained from the National Heart, Lung, and Blood Institute-funded Lung Tissue Research Consortium (25). The diagnosis of IPF was based on American Thoracic Society/European Respiratory Society criteria (3), and all samples displayed the typical pattern of UIP.

Animals

A total of 76 male Sprague Dawley rats with 200–210 g body weight (Harlan Laboratories Ltd., Füllinsdorf, Switzerland)

were anesthetized by inhalation of 5% attane isoflurane (Provet AG, Lyssach, Switzerland); group 1 (saline, $n = 35$) was instilled intratracheally with 1 ml/kg saline solution (0.9% NaCl), and group 2 (bleomycin, $n = 41$) was instilled intratracheally with 1.5 mg/kg bleomycin (Biomol, Hamburg, Germany)/sterile saline (0.9% NaCl). The animals were killed 3, 7, 14, 21, 28, 42, and 56 days after instillation by an injection of thiobutabarbital (Inactin, 100 mg/kg intraperitoneal; Sigma-Aldrich, St. Louis, MO). Lungs were removed and prepared for lung hydroxyproline measurements (right middle lobe), RNA extractions (right cranial lobe), and histopathology (left lobe). Pirfenidone (Beta Pharma Inc., Branford, CT) was administered as food admix (0.5%), and treatment was initiated at Day 7. Pirfenidone-treated and control food-fed animals were killed ($n = 6$ each) for gene expression analysis and histological scoring (Day 14) and after 21 days of treatment ($n = 6$ each) for histological scoring (Day 28). Hydroxyproline content was measured according to published procedures (26). Histological tissue sections were stained and scored for severity in a blinded fashion.

Gene Expression Analysis

Human or rat microarray (Agilent, Santa Clara, CA) experiments were performed according to the manufacturer's protocol. The complete human dataset is available online from the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) with accession number GSE47460, or from the Lung Tissue Research Consortium website (<http://www.ltrcpublic.com>). The rat microarray dataset is available with accession number GSE48455.

Microarray Statistical Data Analysis

Principal component analysis (PCA) was used to reduce the dimensionality of the data, to visually identify trends within our data and to identify outliers. A total of 18 of 73 rats were separated from their respective groups and were thereby classified as experimental outliers and removed from further analysis. ANOVA models were applied to the data using the Bioconductor package, limma (27). The empirical Bayes method from limma was used to compute moderated t statistics and the corresponding P values that were

then corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate (FDR)-controlling procedure (28). Various subsets of patients were selected and submitted to the "annotation-driven split" algorithm (29). Detailed descriptions for microarray statistical analysis and quantitative RT-PCR (QPCR) are provided in the online supplement.

Cell Culture

Totals of 0.3×10^5 A549 and 0.5×10^5 normal primary human lung fibroblasts (NHLFs)/well were seeded in 24-well plates and grown for 24 hours. The cell culture medium was replaced with serum-free growth medium/0.1% fatty acid-free BSA. The next day, the cells were treated with 0.5 mg/ml pirfenidone and/or 10 ng/ml TGF- β 1 (Sigma-Aldrich, St. Louis, MO) for 6 hours and lysed in TriReagent (Life Technologies, Zug, Switzerland). Detailed descriptions are provided in the online supplement.

Results

Temporal Development of Lung Fibrosis in Bleomycin-Treated Animals

The temporal development of lung fibrosis was examined 3, 7, 14, 21, 28, 42, and 56 days after a single intratracheal instillation of bleomycin (1.5 mg/kg) using semiquantitative histological scoring. New collagen synthesis was quantified by measuring hydroxyproline concentrations in lung extracts (Table 1). The selected bleomycin dose did not cause mortality

during the entire study. Reduced body weight of bleomycin-treated animals compared with saline-treated animals was observed during the first 14 days, but not at later time points. Bleomycin-treated animals showed a pronounced inflammatory response in histological examinations that was characterized by substantial inflammatory cell infiltration, with a peak at Day 3. This acute inflammation response resolved, although mild inflammation remained until Day 56. Significantly increased levels of monocyte-derived histiocytes were first seen in lung tissue of bleomycin-treated animals at Day 7, and this finding persisted throughout the entire observation period. First signs of histological fibrosis and elevated hydroxyproline concentrations were seen at Day 7, with a subsequent increase until Day 14. Histological fibrosis scores then remained constant throughout the remaining observation period, whereas hydroxyproline levels steadily increased from Day 14 to Day 56. Histological scoring and hydroxyproline levels in animals killed at Day 21 were lower than those killed on Day 14 or subsequent time points (Days 28, 42, and 56; Table 1; see also Figure E18 in the online supplement).

Whole-Genome mRNA Microarray Analysis

PCA revealed clusters of different animal subgroups. Clusters representing samples taken at Days 3, 7, and 14 were very well separated from the other time points and from each other. The samples taken at Days 21, 28, 42, and 56 were clearly separated from samples taken at earlier

time points, but were less well segregated from each other (Figure 1A). The PCA visualization reflects the temporal evolution of bleomycin-induced lung fibrosis based on gene expression signatures. Linear models, which were fitted to the log₂-transformed expression levels, were next applied to identify differentially expressed genes that were defined by a differential expression greater than 0.7 and an FDR-adjusted *P* value less than 0.05 at each time point. Compared with saline-treated control animals, the highest number of differentially expressed genes (2,459) were found at Day 3 in the bleomycin-treated animals, and fewer differentially expressed genes were found at subsequent time points, namely, 1,892 at Day 7, 1,317 at Day 14, 285 at Day 21, 423 at Day 28, 205 at Day 42, and 299 at Day 56 (Tables E1–E14). Ingenuity pathway analysis was used to interrogate the differentially expressed genes. The number of genes associated with known biological functions indicated two consecutive major response phases (Figure 1B). A first pronounced inflammatory phase, which showed a peak at Day 3 with 447 differentially regulated inflammatory disease-associated genes, was followed by a second phase that was associated with connective tissue disorders at Days 7 and 14. The second phase peaked at Day 14 with 157 differentially regulated connective tissue disorder-associated genes. Beyond Day 14, the majority of genes with increased or decreased expression levels after bleomycin treatment returned to control levels. Very few genes were persistently dysregulated at later time points (i.e., at Days 28, 42, and

Table 1. Summary of the Bleomycin-Induced Rat Lung Fibrosis Time Course Study

	3 Days		7 Days		14 Days		21 Days		28 Days		42 Days		56 Days	
	Control	Bleomycin	Control	Bleomycin	Control	Bleomycin	Control	Bleomycin	Control	Bleomycin	Control	Bleomycin	Control	Bleomycin
Fibrosis score	0	1*	0	2 [†]	0	3 [†]	0	2 [†]	0	3 [†]	0	3 [†]	0	2 [†]
Inflammation	0	4 [†]	0	3 [†]	0	3 [†]	0	1*	0	3 [†]	0	2 [†]	1	2*
Histiocytosis	0	1*	0	2 [†]	1	1	0	2 [†]	0	2 [†]	0	1 [†]	1	3 [†]
Mast cells	1	0	0	1	0	2 [†]	1	2 [†]	0	2 [†]	0	2 [†]	0	2 [†]
Hydroxyproline	0.14	0.11	0.16	0.14	0.19	0.26	0.19	0.22*	0.17	0.34 [†]	0.18	0.35 [†]	0.19	0.45 [†]
Body weight	212	191 [†]	245	212*	291	240 [†]	323	319	345	312	390	350*	418	380
<i>n</i>	5	5	5	5	5	5	5	5	5	7	5	7	5	7

Histological scoring of fibrosis, inflammatory cell infiltration, histiocytosis, and mast cell accumulation according to severity (1 = minimal; 2 = slight; 3 = moderate; 4 = marked; 5 = severe). Median values are indicated (Mann-Whitney test: bleomycin versus control). Hydroxyproline is measured in milligrams per lobe; body weight is measured in grams.

*0.01 < *P* < 0.05.

[†]*P* < 0.01.

Figure 1. Gene expression analysis of bleomycin-induced rat lung samples segregate different temporal phases in the development of lung fibrosis. (A) The first two principal components (PC1 and PC2) of this principal component analysis (PCA) account for more than 60% of the total variance, and are used to visualize relative animal positions in the gene expression-defined space throughout the time course experiment. (B) Ingenuity pathway analysis identified different response phases after bleomycin administration. The number of differentially expressed genes with known biological activity (inflammatory disease and connective tissue disorder) is indicated (y axis) for each time point after bleomycin administration (x axis). The top 20 dysregulated genes are indicated for each response phase peak (Days 3 and 14).

56), and several of those were associated with wound-healing responses, including chemokine (C-C motif) ligand 7 binding (*Ccl7*) and its receptor (*Ccr1*), complement factor I (*Cfi*), complement factor D (*Cfd*), chymase 1 (*Cma1*), chemokine (C-C motif) ligand 2 (*Ccl2*), secreted phosphoprotein 1 (*Spp1*; osteopontin), IL8 receptor (*Il8R*), and tachykinin precursor 1 (*Tac1*).

Comparison with Human IPF-Derived Genomics Data

A systematic comparative analysis of gene expression changes at different time points of the bleomycin-induced animal model and genomics data from patients with IPF was performed to determine the time point of highest molecular congruence, and to identify shared molecular signatures. A total of 1,696 differentially expressed genes were identified in lung samples from patients with IPF compared with control lung samples. In comparison to the differentially expressed genes that were identified at different time points of the bleomycin-induced animal model, 584 common differentially expressed genes were identified at Day 3, 468 at Day 7, 337 at Day 14, 76 at Day 21, 103 at Day 28, 55 at Day 42, and 89 at Day 56. The most significant common differentially expressed genes were identified based on a differential expression greater than 0.7

on a log₂ scale and FDR-adjusted *P* value less than 0.05. This analysis revealed that the bleomycin-induced rat lung samples and human IPF lung samples displayed

the highest degree of similarity at Days 7 and 14, although maximal gene expression change for the majority of the common dysregulated genes in the

Figure 2. Scatter plot showing differentially expressed genes of the bleomycin-treated rat lung fibrosis model at Day 7 and patients with idiopathic pulmonary fibrosis (IPF). (A) x and y axis represent the log₂ fold gene expression change in IPF and bleomycin-treated rats against their respective controls. The red lines represent the positive correlation between the bleomycin-treated rat and IPF. The black lines represent no expression change in either bleomycin-treated rat or IPF. The genes that are labeled in red displayed the most similar fold differential expression in bleomycin-treated rats and IPF. Genes with significant expression changes in IPF, but no change in bleomycin-treated rats, are indicated in dark blue. Genes with unique change in bleomycin-treated rats, but no change in IPF, are indicated in green. (B) A detailed view of the upper right quadrant of (A), indicating highly conserved, up-regulated genes in bleomycin-treated rat and IPF. Among the most significantly up-regulated genes were *Mmp7/MMP7*, *Pla2 g2a/PLA2G2A*, *C6/C6*, *Lcn2/LCN2*, and *Cthrc1/CTHRC1*, as indicated.

Table 2. Common Up-Regulated Genes in Idiopathic Pulmonary Fibrosis Lungs and Bleomycin-Induced Rat Lungs at Day 7

Symbols	Name	log ₂ (fc) IPF	Adjusted <i>P</i> Value: IPF	log ₂ (fc) BLEO	Adjusted <i>P</i> Value: BLEO
Mmp7/MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	3.1852	0.0000	4.3751	0.0000
Pla2g2a/PLA2G2A	Phospholipase A2, group IIA (platelets, synovial fluid)	2.9701	0.0000	2.0732	0.0000
Lcn2/LCN2	Lipocalin 2	2.2327	0.0000	1.8141	0.0000
Cthrc1/CTHRC1	Collagen triple helix repeat containing 1	2.0630	0.0000	1.6624	0.0000
C6/C6	Complement component 6	1.9342	0.0000	2.6717	0.0001
Ctse/CTSE	Cathepsin E	1.7612	0.0000	1.0269	0.0000
Dclk1/DCLK1	Double cortin-like kinase 1	1.6314	0.0000	0.8804	0.0000
Anln/ANLN	Anillin, actin binding protein	1.4936	0.0000	0.9953	0.0000
Kcnn4/KCNN4	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	1.4457	0.0000	1.2438	0.0000
Aspn/ASPN	Asporin	1.3728	0.0000	0.8549	0.0002
Pkib/PKIB	Protein kinase (cAMP-dependent, catalytic) inhibitor β	1.3684	0.0000	0.9389	0.0000
Fhl2/FHL2	Four and a half LIM domains 2	1.3629	0.0000	0.7183	0.0000
Mnd1/MND1	Meiotic nuclear divisions 1 homolog (<i>Saccharomyces cerevisiae</i>)	1.3085	0.0000	0.9863	0.0000
Mycn/MYCN	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	1.3002	0.0000	0.9627	0.0001
Calca/CALCA	Calcitonin-related polypeptide α	1.2624	0.0000	0.8264	0.0011
Slc2a5/SLC2A5	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	1.2436	0.0000	1.6476	0.0000
Fkbp11/FKBP11	FK506 binding protein 11, 19 kD	1.2108	0.0000	0.8330	0.0000
Gdf15/GDF15	Growth differentiation factor 15	1.1997	0.0000	0.7630	0.0000
Gal/GAL	Galanin prepropeptide	1.1803	0.0000	1.2269	0.0002
Top2a/TOP2A	Topoisomerase (DNA) II α , 170 kD	1.1063	0.0000	1.2323	0.0000
Tmem213/TMEM213	Transmembrane protein 213	1.0949	0.0003	1.6466	0.0000
Podnl1/PODNL1	Podocan-like 1	1.0510	0.0000	1.4370	0.0000
Pln/PLN	Phospholamban	1.0086	0.0000	0.9020	0.0005
Mia/MIA	Melanoma inhibitory activity	1.0037	0.0000	0.9829	0.0001
Bik/BIK	BCL2-interacting killer (apoptosis inducing)	0.9539	0.0000	1.0432	0.0000
Col1a2/COL1A2	Collagen, type I, α 2	0.9508	0.0000	1.0491	0.0000
Ccnb2/CCNB2	Cyclin B2	0.9278	0.0000	1.0869	0.0000
MGC105649/C15orf48	Chromosome 15 open reading frame 48	0.9174	0.0000	0.8841	0.0003
Ptges/PTGES	Prostaglandin E synthase	0.8984	0.0000	0.8849	0.0000
Ctsk/CTSK	Cathepsin K	0.8796	0.0000	1.5412	0.0000
Nuf2/NUF2	NUF2, NDC80 kinetochore complex component, homolog (<i>S. cerevisiae</i>)	0.8425	0.0000	1.4879	0.0000
Bub1b/BUB1B	Budding uninhibited by benzimidazoles 1 homolog β (yeast)	0.8123	0.0000	1.1927	0.0000
Fap/FAP	Fibroblast activation protein, α	0.8039	0.0000	1.4624	0.0000
Col5a1/COL5A1	Collagen, type V, α 1	0.7904	0.0000	1.0771	0.0000
Fkbp10/FKBP10	FK506 binding protein 10, 65 kD	0.7875	0.0000	0.8553	0.0000
Uchl1/UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	0.7765	0.0000	0.8931	0.0000
Pla2g7/PLA2G7	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	0.7526	0.0001	1.2517	0.0000
Spc25/SPC25	SPC25, NDC80 kinetochore complex component, homolog (<i>S. cerevisiae</i>)	0.7520	0.0000	1.1505	0.0000
Mlf1ip/MLF1IP	MLF1 interacting protein	0.7504	0.0000	0.7859	0.0014
Sel1l3/SEL1L3	sel-1 suppressor of lin-12-like 3 (<i>Caenorhabditis elegans</i>)	0.7333	0.0000	1.3812	0.0000
Foxm1/FOXM1	Forkhead box M1	0.7288	0.0000	0.8459	0.0000

Definition of abbreviations: BLEO, bleomycin; fc, fold change; IPF, idiopathic pulmonary fibrosis.

bleomycin model occurred at Day 7. Further analysis therefore focused on the common differentially expressed genes

at Day 7 after bleomycin-induced lung fibrosis. Remarkably, similar expression change intensities were found for several

common differentially expressed genes in both species (Figure 2, Tables 2 and 3), such as matrix metalloproteinase 7

Table 3. Common Down-Regulated Genes in Idiopathic Pulmonary Fibrosis Lungs and Bleomycin-Induced Rat Lungs at Day 7

Symbols	Name	log ₂ (fc) IPF	Adjusted P Value: IPF	log ₂ (fc) BLEO	Adjusted P Value: BLEO
Esm1/ESM1	Endothelial cell-specific molecule 1	−2.0894	0.0000	−2.5308	0.0000
Tmem100/TMEM100	Transmembrane protein 100	−2.0296	0.0000	−2.4067	0.0000
Stxbp6/STXBP6	Syntaxin binding protein 6 (amisyn)	−1.9271	0.0000	−1.6196	0.0000
Gcom1/GCOM1	GRINL1A complex locus 1	−1.8879	0.0000	−0.9542	0.0000
Hpgd/HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	−1.6276	0.0000	−1.9077	0.0000
Vegfa/VEGFA	Vascular endothelial growth factor A	−1.5763	0.0000	−1.4624	0.0000
Mme/MME	Membrane metallo-endopeptidase	−1.5015	0.0000	−1.1953	0.0000
Emp2/EMP2	Epithelial membrane protein 2	−1.4698	0.0000	−1.1511	0.0000
Slc1a1/SLC1A1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	−1.3418	0.0000	−0.7041	0.0001
Clic5/CLIC5	Chloride intracellular channel 5	−1.3321	0.0000	−0.8087	0.0151
Ptprr/PTPRR	Protein tyrosine phosphatase, receptor type, R	−1.3184	0.0000	−0.8067	0.0002
Anxa3/ANXA3	Annexin A3	−1.2812	0.0000	−0.7934	0.0000
Lrrn3/LRRN3	Leucine rich repeat neuronal 3	−1.2783	0.0000	−1.9112	0.0000
Rapgef5/RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5	−1.2716	0.0000	−0.9431	0.0016
Olfml2a/OLFML2A	Olfactomedin-like 2A	−1.2472	0.0000	−0.7184	0.0000
Sgef/ARHGEF26	Rho guanine nucleotide exchange factor (GEF) 26	−1.2272	0.0000	−0.8600	0.0001
Sdpr/SDPR	Serum deprivation response	−1.2015	0.0000	−0.8812	0.0000
Adrb2/ADRB2	Adrenoceptor β 2, surface	−1.1906	0.0000	−0.7065	0.0005
Ramp2/RAMP2	Receptor (G protein-coupled) activity modifying protein 2	−1.1806	0.0000	−0.7193	0.0001
Ccdc68/CCDC68	Coiled-coil domain containing 68	−1.1295	0.0000	−1.0026	0.0000
RGD1306437/C13orf1	NA	−1.1262	0.0000	−0.9167	0.0000
Cav2/CAV2	Caveolin 2	−1.1061	0.0000	−1.4034	0.0000
Npr3/NPR3	Natriuretic peptide receptor C/guanylate cyclase C	−1.0721	0.0000	−1.6777	0.0000
Tal1/TAL1	T cell acute lymphocytic leukemia 1	−1.0642	0.0000	−0.8062	0.0000
Lifr/LIFR	Leukemia inhibitory factor receptor α	−1.0560	0.0000	−0.9442	0.0000
Prkce/PRKCE	Protein kinase C, ε	−1.0289	0.0000	−1.1969	0.0000
Cav1/CAV1	Caveolin 1, caveolae protein, 22 kD	−1.0229	0.0000	−1.1039	0.0000
RGD1311307/C6orf145	NA	−0.9754	0.0000	−0.8753	0.0000
Nebi/NEBL	Nebulette	−0.9688	0.0000	−1.0265	0.0011
Nedd9/NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	−0.9685	0.0000	−0.9556	0.0000
S1pr5/S1PR5	Sphingosine-1-phosphate receptor 5	−0.9515	0.0000	−0.8378	0.0011
Afap111/AFAP1L1	Actin filament associated protein 1-like 1	−0.9385	0.0000	−0.8788	0.0000
Thbd/THBD	Thrombomodulin	−0.9359	0.0000	−0.9530	0.0000
Pard6b/PARD6B	Par-6 partitioning defective 6 homolog β (<i>C. elegans</i>)	−0.9315	0.0000	−1.0295	0.0000
Radil/RADIL	Ras association and DIL domains	−0.9314	0.0000	−0.8165	0.0001
Dnase2b/DNASE2B	DeoxyRNase II β	−0.9150	0.0000	−1.0055	0.0029
LOC691221/C5orf4	Chromosome 5 open reading frame 4	−0.8983	0.0000	−0.7754	0.0000
Sh3bp5/SH3BP5	SH3-domain binding protein 5 (BTK-associated)	−0.8774	0.0000	−0.7770	0.0000
Fgg/FGG	Fibrinogen γ chain	−0.8564	0.0435	−1.1679	0.0000
Epb4.115/EPB41L5	Erythrocyte membrane protein band 4.1-like 5	−0.8472	0.0000	−0.9102	0.0000
Tspan12/TSPAN12	Tetraspanin 12	−0.8171	0.0000	−1.0108	0.0000
Slc4a1/SLC4A1	Solute carrier family 4, anion exchanger, member 1 (erythrocyte membrane protein band 3, Diego blood group)	−0.8167	0.0000	−1.2904	0.0000
Zfp365/ZNF365	Zinc finger protein 365	−0.7955	0.0000	−1.1630	0.0000
Phactr1/PHACTR1	Phosphatase and actin regulator 1	−0.7675	0.0000	−1.3533	0.0000
Gpd1/GPD1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	−0.7449	0.0000	−0.8036	0.0000

(Continued)

Table 3. (Continued)

Symbols	Name	log ₂ (fc) IPF	Adjusted <i>P</i> Value: IPF	log ₂ (fc) BLEO	Adjusted <i>P</i> Value: BLEO
Vep1/VEPH1	Ventricular zone expressed PH domain homolog 1 (zebrafish)	−0.7209	0.0000	−0.9310	0.0001
Selenbp1/ SELENBP1	Selenium binding protein 1	−0.7038	0.0000	−0.9965	0.0000

Definition of abbreviations: BLEO, bleomycin; fc, fold change; IPF, idiopathic pulmonary fibrosis.

(MMP7/*Mmp7*; 9.2-fold in IPF, 21.1-fold in bleomycin), the phospholipase A2, group 2A (*PLA2G2A/Pla2 g2a*; 7.8-fold in IPF, 4.3-fold in bleomycin), lipocalin-2 (*LCN2/Lcn2* 4.6-fold in IPF, 3.5-fold in bleomycin), collagen triple helix repeat containing 1 (*CTHRC1/Cthrc1*; 4.3-fold in IPF, 3.2-fold in bleomycin), and complement component 6 (*C6*; 3.7-fold in IPF, 6.5-fold in bleomycin).

Ingenuity pathway analysis classification revealed enrichment of several pathways and functional groups among common up-regulated genes. For instance, *PLA2G2A/Pla2 g2a*, phospholipase A2, group VII (*PLA2G7/Pla2 g7*), prostaglandin E synthase (*PTGES/Ptges*), and glycerol-3-phosphate dehydrogenase 1 (*GPD1/Gpd1*) are eicosanoid signaling components, suggesting that the dysregulation of this particular pathway is well conserved between the animal model and IPF, a finding of significant interest considering the phenotype of prostaglandin synthase 2 knockout mice (30). Other common differentially expressed genes, such as *MMP7/Mmp7*, *CTHRC1/Cthrc1*, vascular endothelial growth factor A (*VEGFA/Vegfa*), asporin (*ASPN/Aspn*), collagen type I $\alpha 2$ (*COL1A2/Col1a2*), and collagen type V $\alpha 1$ (*COL5A1/Col5a1*), are associated with extracellular matrix responses. *C6*, caveolin 1 (*CAV1/Cav1*), fibrinogen γ chain (*FGG/Fgg*), and thrombomodulin (*THBD/Thbd*) were also found to be common dysregulated genes, and are components of the complement and coagulation cascades.

A number of genes that were either up- or down-regulated in human samples were not found to be differentially expressed in bleomycin-treated animals (Tables E15 and E16). This included several genes that were associated with extracellular matrix formation, such as collagen type V $\alpha 2$ (*COL5A2*), collagen type 10 $\alpha 1$ (*COL10A1*), collagen type 15 $\alpha 1$ (*COL15A1*), *MMP3*, *MMP10*, *MMP11*, prolyl 4-hydroxylase α

polypeptide III (*P4HA3*), and plasminogen (*PLG*), whereas others, such as cadherins 2 and 3 (*CDH2* and *CDH3*) and claudins 8 and 14 (*CLDN8* and *CLDN14*), were linked to cell junction organization. Interestingly, a number of genes that were only found in IPF are related to the regulation of organ morphogenesis, such as smoothened, frizzled family (*SMO*), grainyhead-like 3 (*GRH13*), Wilms tumor 1 (*WT1*), SIX homeobox 1 (*SIX1*), sex determining region Y (*SOX9*), paired box 9 (*PAX9*), nerve growth factor receptor (*NGFR*), bone morphogenic protein 4 (*BMP4*), secreted frizzled-related protein 2 (*SFR2*), and twist homolog 1 (*TWIST1*), suggesting that the aberrant activation of developmental pathways may be unique to human pulmonary fibrosis (10, 31). The reverse findings (i.e., significant gene expression changes in the bleomycin-induced rat lung fibrosis model, but no up-regulation in IPF) were also observed, but these differentially expressed genes were not further investigated due to lack of translational relevance.

The Translational Gene Set Distinguishes Patients with IPF from Control Subjects and Correlates with Disease Severity

Although an increasing number of potential IPF-specific biomarkers have been described (32, 33), there is still no validated prognostic or diagnostic biomarker in clinical use. Lung biopsy gene signatures could be considered, as lung biopsies are often part of the IPF diagnostic procedure. We therefore used annotation-driven split algorithms to identify gene sets within the bleomycin-induced animal time course model that were best able to separate the group of patients with IPF from control subjects based on gene expression patterns. The lowest *P* value of annotation-driven clustering was obtained with a bleomycin-induced animal gene set from Day 7

(*P* = 0.076). However, this gene signature classified some of the control patients as IPF (6 from 108), and some of the patients with IPF were classified as control subjects (18 from 100) (Figure 3A). To identify the minimal set of genes from the bleomycin-induced animal gene signature at Day 7 that was able to differentiate the patients with IPF from control subjects, a PCA was applied. Figure 3B illustrates the genes with the highest loadings on principal component (PC)1 and PC2. We then selected a subgroup of 13 genes that mostly associated with PC1-positive loading (Figure 3C) to see whether this subset of genes was able to segregate IPF from control subjects. This gene set contained the differentially expressed genes, *PLA2G2A/Pla2 g2a*, *CTHRC1/Cthrc1*, *CTSE/Ctse*, *MMP7/Mmp7*, *C6*, *LCN2/Lcn2*, NEL-like 1 (*NELL1/Nell1*), proprotein convertase subtilisin/kexin type 1 (*PCSK1/Pcsk1*), gremlin 1 (*GREM1/Grem1*), Galanin prepropeptide (*GAL/Gal*), solute carrier family 2, member 5 (*SLC2A5/Slc2a5*), four and a half LIM-domains 2 (*FHL2/Fhl2*), and UDP glucuronosyltransferase 1 family, polypeptide A6 (*UGT1A6/Ugt1a6*). A further PCA analysis using control subjects and patients with IPF revealed that this reduced subset of translational genes alone was able to differentiate patients with IPF from control subjects (Figure 3D).

The subset of 13 translational genes was further investigated, and 12 genes (*C6*; *CTHRC1/Cthrc1*; *CTSE/Ctse*; *FHL2/Fhl2*; *GAL/Gal*; *GREM1/Grem1*; *LCN2/Lcn2*; *MMP7/Mmp7*; *NELL1/Nell1*; *PCSK1/Pcsk1*; *PLA2G2A/Pla2 g2a*; *SLC2A5/Slc2a5*) were confirmed to be differentially expressed in the samples from the bleomycin-induced animal model at Day 7 using QPCR analysis (Figure 4). The set of 12 confirmed translational markers equally segregated patients with IPF from control subjects, as the 13 originally identified genes did. In addition, the translational gene set was also able to

Figure 3. PCA of IPF and human control using the bleomycin-treated rat genomic signature at Day 7. (A) The first two PCs separate the control subjects (light blue) from the patients with IPF (green). Based on the rat genomic signature, some of the control subjects were identified as patients with IPF (red) and, similarly, some of the patients with IPF behaved like the control subjects (dark blue). (B) Gene identities that support the separation of IPF and control as shown in A. (C) The minimal translational gene subset from B that can be used in a PCA (D) to differentiate the IPF from the control subjects.

segregate control subjects from patients with IPF in two publicly available, independent clinical IPF/control datasets (GSE10667 and GSE24206), thereby supporting the findings (Figure E17). Furthermore, in patients with IPF, the expression levels of the translational gene markers, *GREM1*, *MMP7*, *CTHRC1*, and *FHL2*, showed a significant negative correlation with percent diffusing capacity of carbon monoxide (%*DL*_{CO}) (*GREM1*, $r = -0.68$, adjusted $P = 4.2e-25$; *MMP7*, $r = -0.64$, adjusted $P = 3.4e-22$; *CTHRC1*, $r = -0.64$, adjusted $P = 3.4e-22$; and *FHL2*, $r = -0.61$, adjusted $P = 1.7e-19$), a parameter associated with disease severity (34, 35). IPF samples obtained from lung

transplantation (category 1, lung explant single; and category 2, lung explant bilateral) showed higher *MMP7* expression and a lower %*DL*_{CO} compared with IPF samples obtained from lung biopsies (category 3, lung lobectomy; or category 4, lung biopsy) (Figure 5). A similar, significant inverse correlation was found between *MMP7* and percent predicted forced vital capacity, but with a lower correlation factor ($r = -0.57$; adjusted $P = 1.9e-18$; data not shown).

Pirfenidone Attenuates Translational Gene Expression Signatures *In Vivo*

The bleomycin-induced lung fibrosis model is often questioned for its value to predict the

potential of novel antifibrotic therapies, and translational biomarkers could improve the use of this model in drug discovery (22). We therefore assessed the effect of pirfenidone, which is an orally active synthetic molecule that has been recently approved for the treatment of IPF in some countries, but not in the United States (36), in the bleomycin-induced lung fibrosis model using a “therapeutic” treatment regimen. Anti-inflammatory and antifibrotic activities of pirfenidone had been previously demonstrated *in vivo* (37) and *in vitro* (38–41). Effects of pirfenidone on the newly identified translational gene subset were monitored by QPCR at Day 14.

Figure 4. Expression levels of the translational gene subset with quantitative RT-PCR (QPCR) confirmed differential expression in the bleomycin-treated rat model at Day 7. The y axis shows the log₂ of gene expression. The box-plot representation shows the median value (center line), the lower and upper limits of each box depicting the first and third quartiles, respectively. The whiskers represent smallest and largest data points. The statistical significance (*P* values using *t* test) between vehicle (VEH) and bleomycin treatment (BLEO) are indicated. Differences are considered significant at: *****P* < 0.0001; ****P* < 0.001; ***P* < 0.01; **P* < 0.05. ns, not significant.

Semiquantitative histological scoring was performed at Days 14 and 28. Of the 12 translational genes that were shown to be differentially expressed at Day 7 in the bleomycin-induced lung fibrosis model, nine genes remained significantly up-regulated at Day 14. The bleomycin-induced gene expression increase of two of the nine genes, namely, *Cthrc1* and *Fhl2*, were attenuated in pirfenidone-treated compared with vehicle-treated animals (Figure 6). Histopathological lung fibrosis scores at Days 14 and 28 were reduced by one severity unit at each time point (*P* < 0.02) in pirfenidone-treated

compared with vehicle-treated animals (Table 4, Figure E19).

Pirfenidone Attenuates Translational Gene Expression *In Vitro*

To investigate the gene expression effects by pirfenidone *in vitro*, we used NHLFs and the human lung epithelial cell line, A549, and stimulated them with TGF-β1. TGF-β1 is a well-known profibrotic mediator, and was previously shown to induce myofibroblast differentiation and epithelial-mesenchymal transition. Both processes are considered important contributors during pathological fibrosis

(5). We first quantified gene expression changes of the translational gene set in NHLFs and A549 cells at 3, 6, and 24 hours after stimulation with TGF-β1, and observed that the expression levels of *CTHRC1*, *FHL2*, and *GAL* in NHLFs and *CTHRC1*, *FHL2*, *GAL*, *GREM1*, and *SCL2A5* in A549 cells had already significantly increased at 6 hours after treatment initiation (data not shown). This demonstrated that some members of the translational gene set were expressed in these two cellular systems and were differentially regulated by TGF-β1. The effects of pirfenidone were therefore

Figure 5. Gene expression changes of *MMP7*, *FHL2*, *GREM1*, and *CTHRC1* and percent diffusing capacity of carbon monoxide (%DL_{CO}) segregate patients according to disease severity. Expression levels of the 12 translational genes exhibit a negative Pearson correlation coefficient with the %DL_{CO}. The patients are classified according to the tissue type (category 1, patients with IPF that received transplantation of a single lung [lung explant single]; category 2, both lungs [lung explants bilateral]; category 3, lobectomy/wedge resection; and category 4, biopsy).

examined 6 hours after concomitant incubation with TGF- β 1 to capture influences on early gene expression changes. Pirfenidone significantly inhibited the TGF- β 1-induced increase of *CTHRC1* in NHLFs (Figure 7A) and led to a pronounced attenuation of TGF- β 1-mediated gene expression increases of *CTHRC1*, *GAL*, and *GREM1* in A549 epithelial cells (Figures 7B–7D).

Discussion

The most commonly used animal model to investigate potential novel antifibrotic therapies for IPF is the rodent bleomycin-induced lung fibrosis model. In the past years, several compounds have shown efficacy in animal models, but the majority of those tested in clinical trials failed to show significant therapeutic effects in patients with IPF (23, 42, 43). We thus performed a systematic genomic comparison of the

bleomycin-induced rat lung fibrosis model using a time course experiment and human IPF patient-derived samples to better characterize this animal model, and to identify novel biomarkers with translational value. The results of our studies show that: (1) congruence between the animal model and IPF at the genomic level was most pronounced 7 days after bleomycin instillation; (2) genomic signatures of many, but not all, biological processes were conserved in patients with IPF and bleomycin-induced animals; (3) the expression changes of a novel, minimal subset of genes with translational significance were able to segregate patients with IPF from control subjects; (4) the expression changes of four genes of this novel translational gene panel, in combination with %DL_{CO}, stratified patients with IPF according to disease severity; and (5) pirfenidone was able to attenuate the expression changes of some of these translational marker genes *in vitro*

and in the bleomycin-induced lung fibrosis model.

In our bleomycin-induced rat lung fibrosis time course experiment, the transcriptomic analysis revealed a pronounced acute inflammatory response that was followed by a fibrotic phase, and these findings are highly consistent with previous reports describing this model (22, 23). Histological signs of fibrosis first appeared at Day 7, peaked at Day 14, and no signs of fibrosis severity reversal were seen during the 56-day observation period. A continuous increase in lung tissue hydroxyproline content was detected from Day 14 to Day 56. These findings are reminiscent of the chronic disease progression in IPF, and are similar to the findings described in recently published studies using bleomycin-treated mice (44–46). Despite persisting fibrosis severity and continuously increasing hydroxyproline levels, we found that the number of genes with significant expression

Figure 6. Expression levels of the set of 12 translational genes in the bleomycin rats at Day 14 with and without pirfenidone treatment, as measured by QPCR. The y axis shows the log₂ of gene expression. The box-plot representation shows the median value (*center line*), the lower and upper limits of each box depicting the first and third quartiles, respectively. The whiskers represent smallest and largest data points. The differences between vehicle and bleomycin and bleomycin with and without pirfenidone (BLEO+P and BLEO) are considered significant at: **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. ns, not significant.

changes in bleomycin-treated animals compared with control was dramatically decreased after Day 28. These data may indicate that, once the molecular

mechanisms that trigger the fibrosis response are established, only a minimal number of molecular changes are necessary to maintain a fibrogenic process. Similar

conclusions were recently made based on findings in bleomycin-treated mice (47). The persistence of mast cell markers, such as *Cma1*, in the time course of our bleomycin

Table 4. Summary of the Bleomycin-Induced Rat Lung Fibrosis Study and Pirfenidone Treatment

Therapeutic Treatment	14 Days			28 Days		
	Control	Bleomycin	Pirfenidone	Control	Bleomycin	Pirfenidone
Fibrosis score	0	3 [†]	2*	0	3 [†]	2 [†]
Inflammation	0	3 [†]	3	0	2 [†]	1.5
Histiocytosis	0	2 [†]	2	0	2 [†]	1.5
Hydroxyproline, mg/lobe	0.19	0.27 [†]	0.34	0.22	0.35 [†]	0.33
Body weight, g	303	261 [†]	253	366	335 [†]	311
n	12	12	6	12	12	6

Histological scoring of fibrosis, inflammatory cell infiltration and histiocytosis according to severity (1 = minimal; 2 = slight; 3 = moderate; 4 = marked; 5 = severe). Median values are indicated (Mann-Whitney test: bleomycin versus control and pirfenidone versus bleomycin).

* $0.01 < P < 0.05$.

[†] $P < 0.01$.

[‡] $P < 0.001$.

Figure 7. Relative gene expression levels in transforming growth factor (TGF)- β 1-stimulated normal primary human lung fibroblasts (NHLFs) and A549 cells and influence of pirfenidone. The box-plot representation shows the median value of $\log_2$ of gene expression (center line), the lower and upper limits of each box depicting the first and third quartiles, respectively. The whiskers represent smallest and largest data points. NHLFs or A549 cells were incubated with vehicle (VEH), TGF- β 1 (TGFB), TGF- β 1 with pirfenidone (TGFB + PIRF), or pirfenidone alone (PIRF), and gene expression changes were quantified using QPCR after 6 hours. Genes with expression levels that were significantly up-regulated by TGF- β 1 and attenuated by pirfenidone are shown in NHLFs (A) and A549 cells (B–D). The differences between conditions are considered significant at: **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ ($n = 4$), as indicated. ns, not significant.

model correlated well with the continuous increase in collagen production. An increase of mast cells has been reported in the airways of patients with IPF (48, 49), and the density of mast cells was recently shown to be higher in IPF lung than in other fibrotic lung disease (50). Interestingly, patients with IPF that had a high mast cell density had a higher degree of fibrosis, and the number of mast cells correlated negatively with patient lung function (51).

We compared the microarray time course expression data in the bleomycin model with microarray data from human IPF biopsies to identify molecular markers with translational relevance, and demonstrated that the highest similarity of gene expression between bleomycin-induced lung fibrosis and IPF in animals occurs at 7 days after bleomycin administration. At this time point, the acute inflammatory response had already strongly decreased, and the first profibrotic signatures appeared. This finding provides

additional support that potential antifibrotic compounds should be first administered around this transitory time point to reflect the clinical situation. Several pathways, such as eicosanoid signaling, were dysregulated in bleomycin-induced rat lung fibrosis and IPF. Furthermore, several of the gene expression changes that were unique to patients with IPF and that were not detected in the bleomycin-induced lung fibrosis model were still part of biological processes that are also induced in rats, suggesting that many biological responses that drive IPF disease, but not necessarily all genes, are reflected in the animal model at the transcriptional level.

With this comprehensive comparative genomic analysis, we could identify a minimal translational gene set that represents a phenotypic-disease relationship between the bleomycin-induced rat lung fibrosis model and human IPF. This panel contained 12 genes, namely, *C6*, *Cthrc1*, *Ctse*, *Fhl2*, *Gal*, *Grem1*, *Lcn2*, *Mmp7*, *Nell1*,

Pcsk1, *Pla2 g2a*, and *Slc2a5*, which were all differentially expressed in rat lung tissue at Day 7. Nine of the 12 genes remained significantly up-regulated until Day 14 after bleomycin administration (*C6*, *Cthrc1*, *Fhl2*, *Gal*, *Grem1*, *Lcn2*, *Mmp7*, *Pcsk1*, and *Slc2a5*). Interestingly, we identified a number of molecules that were associated directly or indirectly with Wnt/ β -catenin signaling, including *Mmp7*, *Fhl2*, *Cthrc1*, and *Grem1*, thus emphasizing the potential significance of this pathway in IPF (52–57).

One impressive finding is that the translational gene set allowed us to distinguish IPF lungs from control lungs. However, a small percentage of controls were classified as IPF, and an equally small number of IPF were classified as controls. At present, we can only speculate about the reasons for this “misclassification.” In the case of the small number of patients with IPF that were classified as control subjects, we cannot exclude the possibility that the disease severity at the location of the biopsy, which was used for RNA isolation, was lower than in most other patients with IPF. The reason why some control samples were classified as IPF is more obscure, and it may represent the limitation of our signature or chronic subclinical lung scarring. Remarkably, the gene expression level of four genes (*GREM1*, *MMP7*, *CTHRC1*, and *FHL2*) that belong to this minimal translational gene set, together with %DLCO values, stratified patients with IPF according to disease severity, as reflected by the need for lung transplantation. *MMP7* was previously shown to be a regulator and potentially predictive protein plasma biomarker in IPF (15, 16). Gene expression and immunoreactivity of *GREM1*, a known bone morphogenetic protein signaling inhibitor, were previously shown to be increased in pathogenesis of IPF, and a negative correlation with DLCO corrected for alveolar volume was demonstrated (58). To date, neither *CTHRC1* nor *FHL2* expression levels have been shown to be associated with IPF disease severity. The possibility of applying the translational gene set to distinguish IPF from other interstitial lung diseases, including nonspecific interstitial pneumonia, respiratory bronchiolitis–interstitial lung disease, and hypersensitive pneumonitis, was also investigated. However, the number of biopsies that were available to perform these studies was insufficient to perform

statistical analysis (data not shown). Additional studies are thus warranted to perform further investigations.

The premise of this unbiased and disease-focused translational science approach is the hope that a translational gene panel will provide better preclinical information on the potential of novel therapeutics for IPF. Interestingly, this novel gene set only partially overlaps with well described markers reported in the literature (23, 59–61). To exemplify our translational approach, we used pirfenidone (Esbriet), which is the only currently approved drug for the treatment of IPF, to see how this novel translational gene set responds to drug treatment in the bleomycin-induced rat lung fibrosis model. From the nine differentially expressed genes that were modulated at Day 14 in the bleomycin model, *Crthc1* and *Fhl2*, which are both associated with the TGF- β and Wnt/ β -catenin signaling pathways, were attenuated by pirfenidone. *Crthc1* is expressed by fibroblasts, is involved in vascular remodeling, and decreases collagen matrix deposition (63). *Crthc1* is a downstream target of bone morphogenic protein–SMAD signaling, inhibits TGF- β expression in neointimal lesion formation

(3), and acts as a Wnt cofactor, possibly contributing to the promotion of cell motility (55). *Fhl2* belongs to the FHL protein family of transcriptional cofactors that have been previously associated with fibrogenesis. *Fhl2* is involved in the regulation of pathways associated with cell proliferation, and mice lacking *Fhl2* are characterized by delayed skin wound healing, reduced transcriptional activation of smooth muscle actin, and reduced ability of myofibroblasts to contract (64). To further investigate the translational gene set and the activity of pirfenidone, we employed TGF- β 1-stimulated NHLF and human lung epithelial A549 cells to model myofibroblast differentiation and epithelial–mesenchymal transition, respectively. In these simplified systems, expression changes of several genes of the novel translational gene set were observed and, interestingly, pirfenidone attenuated some of those changes. It is noteworthy that two of the genes that were up-regulated by TGF- β 1 and attenuated by pirfenidone (*CTHRC1* and *GREM1*) were among the genes that correlated with disease severity in our IPF cohort. These findings further support the relevance of the translational gene set, but also suggest that these two

simplified systems do not fully reflect all cellular subtypes or processes that are involved in IPF. They also support our hypothesis that the efficacy of exploratory therapeutic interventions in the complex setting of an *in vivo* bleomycin-induced rat lung fibrosis model could be evaluated using this limited gene set. As pirfenidone was not able to fully normalize all gene expression changes *in vitro* and *in vivo*, additional treatment modalities or combination therapies might be required to fully revert the profibrotic process. Our genomics studies will help to support the optimal use of the bleomycin model on the basis of treatment timing and molecular analysis of translational pathways. Furthermore, this gene set could also find application as a potential diagnostic and prognostic tool for IPF in clinical practice. ■

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